Detection of Monkeypox Virus DNA in Airport Wastewater, Rome, Italy

Giuseppina La Rosa, Pamela Mancini, Carolina Veneri, Giusy Bonanno Ferraro, Luca Lucentini, Marcello Iaconelli, Elisabetta Suffredini

Environmental surveillance can be a complementary tool for detecting pathogens circulating in communities. We detected monkeypox virus DNA in wastewater from Italy's largest airport by using real-time PCR assays targeting the G2R region and *F3L* and *N3R* genes and sequencing. Wastewater surveillance can be quickly adapted to investigate emerging threats.

Monkeypox virus (MPXV), a member of the family *Poxviridae*, causes monkeypox, a viral zoonosis detected in north Africa in the 1970s (*1*). MPXV can be transmitted between humans through contact with lesions, body fluids, respiratory droplets, and contaminated materials (*1*).

In May 2022, an epidemic of monkeypox in nonendemic regions outside Africa began receiving worldwide attention. On July 23, 2022, the World Health Organization declared monkeypox a public health emergency of international concern (*2*), and 24,973 monkeypox cases had been recognized in 45 countries throughout Europe by October 12, 2022 (*3*).

Rapid identification of outbreaks and clusters is critical for infection control. Sewage surveillance has been recognized as a powerful tool for assessing the circulation of pathogens. After the European Union issued Recommendation 2021/472 (http://data.europa.eu/eli/reco/2021/472/oj), wastewater surveillance was successfully used to track SARS-CoV-2 and its variants across EU countries (*4*). Studies have demonstrated MPXV DNA sheds from saliva, feces, urine, semen, and skin lesions (*5*–*7*), suggesting that the viral genome could occur in wastewater. Various research groups involved in SARS-CoV-2 environmental surveillance extended their efforts to investigate MPXV DNA in wastewater. Studies from the Netherlands and western California, USA, have documented successful detection of MPXV DNA in sewage (*8*; M.K. Wolfe et al., unpub. data, https://doi.org/10.1101/2022.07.25.22278043). We investigated whether we could detect MPXV in wastewater in Italy.

Author affiliation: Istituto Superiore di Sanità, Rome, Italy

DOI: https://doi.org/10.3201/eid2901.221311

The Study

We targeted the wastewater treatment plant (WTP) of Italy's largest airport, Fiumicino Airport, in Rome, which had ≈3,000,000 passengers/month during May–July 2022 (https://fiumicinoairport.com/statistics). This WTP has a global capacity of 4,000 m^3 per day. We collected 24-hour composite wastewater samples twice a week during May 30–August 3, 2022, for a total of 20 samples.

Before viral concentration, we pretreated samples in a water bath at 56°C for 30 min to inactivate the virus and protect laboratory technicians, as per a previous study (*9*). We used a polyethylene glycol/sodium chloride precipitation protocol originally developed for SARS-CoV-2 environmental surveillance (*10*,*11*) but modified the protocol by increasing the initial wastewater volume to 90 mL (2 tubes of 45 mL) and eluting all the extracted nucleic acids in 50 μL of elution buffer supplied with the kit. We used NucliSens miniMAG (bioMérieux, https://www.biomerieux.com) semi-automatic extraction platform to extract nucleic acids. We used OneStep PCR Inhibitor Removal Kit (Zymo Research, https://www.zymoresearch.com) to purify DNA.

We used 3 different real-time PCR assays: 2 published in 2004 that target the *N3R* and *F3L* genes (*12*), and 1 developed in 2010 by the US Centers for Disease Control and Prevention, G2R_G generic real-time PCR assay (*13*), which targets the G2R region of the tumor necrosis factor receptor gene. After comparing primers and probes with sequences of the current outbreak, we noted mismatches in primers, probes, or both. Therefore, we designed and tested novel primers and probes that had 100% nucleotide identity with current outbreak sequences, then compared these with the original primers and probes (Table 1). We used MPXV (Slovenia ex Gran

Canaria) DNA (European Virus Archive Global [EVAg]; https://www.european-virus-archive.com) as a control for testing primers and probes (Appendix, https://wwwnc.cdc.gov/EID/article/29/1/22-1311-App1.pdf). We further optimized the assays by evaluating different real-time PCR reagents and primer/probe concentrations (Appendix). We prepared reaction mixes in 25 µL by using TaqPath BactoPure Microbial Detection Master Mix (Thermo Fisher Scientific, https://www.thermofisher.com), 800 mmol of each primer, 500 nmol of the probe, and 5 µL of sample. Amplification conditions included an initial activation step at 95°C for 2 min and 50 cycles of 10 s at 95°C and 30 s at 60°C. We included 10-fold dilutions of the standardized EVAg MPXV DNA (range 740–0.74 copies/µL) in the runs as positive controls and for the rough estimation of viral loads. For each assay, we assessed the limit of detection at 50% (LOD_{50}) on a pure target (i.e., EVAg MPXV DNA) and on MPXV DNA diluted in nucleic acids previously extracted from wastewater samples collected in Europe before monkeypox emerged.

We designed nested PCR assays targeting the same regions as the real-time PCR assays to confirm results by amplicon sequencing using Primer3Plus software

Table 1. Primers and probes used to detect monkeypox virus DNA in airport wastewater, Rome, Italy*

PCR ID	Target	Primer name	Primer ID	Sequence, 5′ → 3′	Position†	Annealing temp. (°C)	Amplicon size	Ref.
1002	G2R	MPVX G F	2368	GGAAAATGTAAAGACAACGAATACAG	194459–84	60	90 bp	(*12*)
		MPVX G R	2369	GCTATCACATAATCTGGAAGCGTA	194525–48			
		MPVX G P	2370	FAM-AAGCCGTAATCTATG TTGTCTATCGTGTCC-BHQ1	194485–514			
1005	G2R	MPVX G F mod	2377	GGAAA**G**TGTAAAGACAACGAATACAG	194459–84	60	90 bp	(*12*)
		MPVX G R mod	2378	GCTATCACATAATCTG**A**AAGCGTA	194525–48			This study
		MPVX G P	2370	FAM-AAGCCGTAATCTATG TTGTCTATCGTGTCC-BHQ1	194485–514			
1003	*F3L*	F3L-F290	2371	CTCATTGATTTTTCGCGGGATA	46313–34	60	107 bp	(*11*)
		F3L-R396	2372	GACGATACTCCTCCTCGTTGGT	46398–419			
		F3Lp333S-MGB	2373	FAM-CATCAGAATCTGTAGGCCGT-MGBNFQ	46398–419			
1008	*F3L*	F3L-F290	2371	CTCATTGATTTTTCGCGGGATA	46313–34	60	107 bp	(*11*)
		F3L-R396 mod	2384	**A**ACGATACTCCTCCTCGTTGGT	46398–419			This study
		F3Lp333S-MGB	2373	FAM-CATCAGAATCTGTAGGCCGT-MGBNFQ	46398–419			
1004	*N3R*	N3R-F319	2374	AACAACCGTCCTACAATTAAACAACA	190641–66	60	139 bp	(*11*)
		N3R-R457	2375	CGCTATCGAACCATTTTTGTAGTCT	190755–79			
		N3Rp352S-MGB	2376	FAM-TATAACGGCGAAGAATATACT-MGBNFQ	190674–94			
1016	*N3R*	N3R-F319	2374	AACAACCGTCCTACAATTAAACAACA	190641–66	60	139 bp	(*11*)
		N3R-R457	2375	CGCTATCGAACCATTTTTGTAGTCT	190755–79			This study
		N3Rp352S-MGB mod	2381	FAM-TATAACGGCGA**C**GAATATACT-MGBNFQ	190674–94			
1006	G2R	G2R-1st cycle F	2379	ATAGCACCACATGCACCATC	194435–54	63	156 bp	This study
		G2R-1st cycle R	2380	AAAGGTATCCGAACCACACG	194590–71			
1005	G2R	MPVX G F mod	2377	GGAAAGTGTAAAGACAACGAATACAG	194459–84	61	90 bp	This study
		MPVX G R mod	2378	GCTATCACATAATCTGAAAGCGTA	194525–48			
1009	*F3L*	F3L-1st cycle F	2385	CAGGGTTAACACCTTTCCAA	46242–61	61	212 bp	This study
		F3L-1st cycle R	2386	TGATCTTCAACGTAGTGCTATGG	46453–31			
1008	*F3L*	F3L-F290	2371	CTCATTGATTTTTCGCGGGATA	46313–34	62	107 bp	(*11*)
		F3L-R396 mod	2384	AACGATACTCCTCCTCGTTGGT	46398–419			This study
1007	*N3R*	N3R-1st cycle F	2382	TCTATCTCGTTCATGGTCGGTAAT	190503–26	64	455 bp	This study
		N3R-1st cycle R	2383	CGCACTGTCTTATTCGCCATT	190957–37			
1004	*N3R*	N3R-F319	2374	AACAACCGTCCTACAATTAAACAACA	190641–66	64	139 bp	(*11*)
		N3R-R457	2375	CGCTATCGAACCATTTTTGTAGTCT	190755–79			

*Bold underlined text in bases represents modifications to the original primers and probes. ID, identification; F, forward; mod, modified; MPXV, monkeypox virus; R, reverse; ref., reference.
†Position based on monkeypox virus reference isolate MPXV_USA_2022_MA001, complete genome, GenBank accession no. ON563414.

Table 2. Wastewater sample results detecting monkeypox virus DNA in airport wastewater, Rome, Italy*

Sample ID	Collection date	Real-time RT-PCR (Cq values)			Nested RT-PCR		
		G2R	F3L	N3R	G2R	F3L	N3R
4419	2022 May 30	–	–	–	–	–	–
4420	2022 Jun 1	–	–	–	–	–	–
4421	2022 Jun 6	–	–	–	–	–	–
4422	2022 Jun 8	–	–	–	–	–	–
4444	2022 Jun 13	–	–	–	–	–	–
4445	2022 Jun 15	+ (40.18)	+ (39.59)		**+**	–	–
4453	2022 Jun 20	–	–	–	–	–	–
4454	2022 Jun 22	–	–	–	–	–	–
4460	2022 Jun 27	–	–	–	–	–	–
4461	2022 Jun 29	–	–	–	–	–	–
4474	2022 Jul 4	–	–	–	–	–	–
4475	2022 Jul 6	–	–	–	–	–	–
4476	2022 Jul 11	–	–	–	–	–	–
4477	2022 Jul 13	–	–	–	–	–	–
4478	2022 Jul 18	+ (38.37)	–	–	+	+	–
4479	2022 Jul 20	–	–	–	+	**+**	–
4480	2022 Jul 25	–	–	–	–	–	–
4481	2022 Jul 27	–	–	–	–	–	–
4482	2022 Aug 3	–	–	–	–	–	–
4483	2022 Aug 1	–	–	–	–	–	–

*Bold positive font (**+**) indicates sequence failure due to insufficient DNA target; amplification band of the expected length was confirmed by duplicate experiments. ID, identification; RT-PCR, reverse transcription PCR; –, negative; +, positive.

(https://www.primer3plus.com) (Table 1). We performed reactions by using 1 µL of 10 µmol primer and 2 µL of sample, and Platinum SuperFi II Green PCR Master Mix (Thermo Fisher Scientific) in a final volume of 25 µL. PCR amplicons on both strands were sequenced by Bio-Fab Research (https://www.biofabresearch.com).

All real-time PCR assays successfully amplified the EVAg MPXV DNA. Compared with the original assay, the modified G2R_G assay showed a decrease in the average quantification cycle (Cq) values of 1.34 cycles (21.93 vs. 23.28), demonstrating a better performance. Therefore, we performed subsequent optimization activities and screening of wastewater samples by using the F3L and N3R assays as originally designed but modified the G2R_G assay for our study.

On pure MPXV DNA, the real-time F3L assay had an LOD_{50} of 0.21 copies/µL, the N3L assay had an LOD_{50} of 0.31 copies/µL, and G2R_G had an LOD_{50} of 0.21 copies/µL. For nucleic acids extracted from sewage samples spiked with MPXV, F3L had an LOD_{50} of 0.43 copies/µL and 2.16 copies/reaction, N3L had an LOD50 of 0.33 copies/µL and 1.65 copies/reaction, and G2R_G had an LOD_{50} of 0.31 copies/µL and 1.55 copies/reaction (Appendix).

Cq values ranged from 38.37–40.18 for 2 wastewater samples that tested positive by real-time PCR (Table 2), indicating relatively low DNA concentrations in the tested samples. Consensus sequences found 100% similarity by BLAST analysis between study sequences and MPXV strains available in GenBank (accession no. OX248696), thus confirming the presence of MPXV DNA.

Conclusions

A crucial aim of infectious disease surveillance is early detection of cases, outbreaks, and clusters, which is essential for disease control. We explored possible methods for monitoring MPXV through wastewater surveillance, a well-established complementary epidemiologic tool used successfully for viral infectious diseases, including SARS-CoV-2 and polio.

Monkeypox prevalence in the general population was low at the time of sample collection, only 20 cases had been detected in Italy as of May 30, 2022. Thus, to maximize the probability of positive samples among those collected, we tested wastewater samples from a large transportation hub, through which millions of persons travel to and from numerous countries. Because harmonized methods for detecting MPXV in wastewater are not yet available, we tested 3 different real-time PCR assays previously designed for clinical samples. We modified the assays by introducing changes in the primer and probe sequences to mitigate the effect of nucleotide mismatches. Among 20 samples, 3 tested positive for MPXV by real-time or nested PCR and sequencing.

In the next stage, we will test wastewater samples from WTPs enrolled in official SARS-CoV-2 environmental surveillance throughout Italy, to map the geographic distribution of MPXV in the country. Further research efforts should focus on elucidating how detection of viral DNA in sewage can be related to reported and confirmed cases. Factors affecting MPXV detection in wastewater also should be studied, including routes and duration of

virus shedding by infected persons, environmental persistence, and analytical sensitivity of the methods used (*14*).

In conclusion, we adapted SARS-CoV-2 wastewater surveillance for MPXV detection in a large airport WTP. Our methods can be applied to wastewater-based epidemiology for monkeypox outbreaks and provides basic tools, including analytic methods. Wastewater surveillance can be rapidly adapted to detect emerging threats, including monkeypox.

This article was preprinted at https://medrxiv.org/cgi/content/short/2022.08.18.22278932v1.

Acknowledgments

We gratefully acknowledge the support of Cavina Lorenzo and Eleuteri Silvia, Aeroporti di Roma (ADR) Fiumicino Airport for wastewater sample collection. We also thank Claudia Del Giudice, Lidia Orlandi and Serena Maccaroni for their technical assistance.

This research was partially supported by collaboration agreement EC G.A. no. 060701/2021/864481/SUB/ ENV. C2, Support to Member States for the creation of systems, local collection points and digital infrastructures for monitoring COVID 19 and its variants in wastewater, Italy.

This publication was supported by the European Virus Archive Global (EVA-GLOBAL) project, which provided the monkeypox DNA for testing real-time PCR assays.

About the Author

Dr. La Rosa is an environmental virologist at the Department of Environment and Health, National Institute of Health (Istituto Superiore di Sanità), Rome, Italy. Her primary research interest is viral pathogen surveillance through the monitoring of sewer systems.

References

1. World Health Organization. Monkeypox 19 May 2022 [cited 2022 Aug 16]. https://www.who.int/news-room/fact-sheets/detail/monkeypox
2. World Health Organization. WHO Director-General declares the ongoing monkeypox outbreak a Public Health Emergency of International Concern, 23 Jul 2022 [cited 2022 Aug 16]. https://www.who.int/europe/news/item/23-07-2022-who-director-general-declares-the-ongoing-monkeypox-outbreak-a-public-health-event-of-international-concern#:~:text=On%20July%2023%2C%20the%20WHO,in%20the%20WHO%20European%20Region
3. European Centre for Disease Control and Prevention - World Health Organization. Joint ECDC-WHO Regional Office for Europe monkeypox surveillance bulletin 12 Oct 2022 [cited 2022 Aug 16]. https://monkeypoxreport.ecdc.europa.eu
4. European Commission. Coronavirus response: monitoring of wastewater contributes to tracking coronavirus and variants across all EU countries 17 Mar 2022 [cited 2022 Aug 16]. https://joint-research-centre.ec.europa.eu/jrc-news/coronavirus-response-monitoring-wastewater-contributes-tracking-coronavirus-and-variants-across-all-2022-03-17_en
5. Peiró-Mestres A, Fuertes I, Camprubí-Ferrer D, Marcos MÁ, Vilella A, Navarro M, et al.; Hospital Clinic de Barcelona Monkeypox Study Group. Frequent detection of monkeypox virus DNA in saliva, semen, and other clinical samples from 12 patients, Barcelona, Spain, May to June 2022. Euro Surveill. 2022;27:2200503. https://doi.org/10.2807/1560-7917.ES.2022.27.28.2200503
6. Lapa D, Carletti F, Mazzotta V, Matusali G, Pinnetti C, Meschi S, et al. INMI Monkeypox Study Group. Monkeypox virus isolation from a semen sample collected in the early phase of infection in a patient with prolonged seminal viral shedding. Lancet Infect Dis. 2022;22:1267–9. https://doi.org/10.1016/S1473-3099(22)00513-8
7. Antinori A, Mazzotta V, Vita S, Carletti F, Tacconi D, Lapini LE, et al.; INMI Monkeypox Group. Epidemiological, clinical and virological characteristics of four cases of monkeypox support transmission through sexual contact, Italy, May 2022. Euro Surveill. 2022;27:2200421. https://doi.org/10.2807/1560-7917.ES.2022.27.22.2200421
8. de Jonge EF, Peterse CM, Koelewijn JM, van der Drift AR, van der Beek RFHJ, Nagelkerke E, et al. The detection of monkeypox virus DNA in wastewater samples in the Netherlands. Sci Total Environ. 2022 Aug 31 [Epub ahead of print]. https://doi.org/10.1016/j.scitotenv.2022.158265
9. La Rosa G, Iaconelli M, Mancini P, Bonanno Ferraro G, Veneri C, Bonadonna L, et al. First detection of SARS-CoV-2 in untreated wastewaters in Italy. Sci Total Environ. 2020;736:139652. https://doi.org/10.1016/j.scitotenv.2020.139652
10. Wu F, Zhang J, Xiao A, Gu X, Lee WL, Armas F, et al. SARS-CoV-2 titers in wastewater are higher than expected from clinically confirmed cases. mSystems. 2020;5:e00614-20. https://doi.org/10.1128/mSystems.00614-20
11. Cutrupi F, Cadonna M, Manara S, Postinghel M, La Rosa G, Suffredini E, et al. The wave of the SARS-CoV-2 Omicron variant resulted in a rapid spike and decline as highlighted by municipal wastewater surveillance. Environ Technol Innov. 2022;28:102667. https://doi.org/10.1016/j.eti.2022.102667
12. Kulesh DA, Loveless BM, Norwood D, Garrison J, Whitehouse CA, Hartmann C, et al. Monkeypox virus detection in rodents using real-time 3′-minor groove binder TaqMan assays on the Roche LightCycler. Lab Invest. 2004;84:1200–8. https://doi.org/10.1038/labinvest.3700143
13. Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods. 2010;169:223–7. https://doi.org/10.1016/j.jviromet.2010.07.012
14. Tiwari A, Adhikari S, Kaya D, Islam MA, Malla B, Sherchan SP, et al. Monkeypox outbreak: wastewater and environmental surveillance perspective. Sci Total Environ. 2022;856:159166. https://doi.org/10.1016/j.scitotenv.2022.159166

Address for correspondence: Giuseppina La Rosa, Department of Environment and Health, Istituto Superiore di Sanità, Viale Regina Elena 299, Rome 00161, Italy; email: giuseppina.larosa@iss.it

Article DOI: https://doi.org/10.3201/eid2901.221311

Detection of Monkeypox Virus DNA in Airport Wastewater, Rome, Italy

Appendix

Comparison of Primers and Probe Sets

To select the primer and probe sets providing better real-time PCR results, a 10^{-2} dilution of the Monkeypox DNA Slovenia ex Gran Canaria, Ref-SKU: 005N-04716 provided by EVAg was tested as control, using PCR IDs 1002, 1003, 1004, 1005, 1008, 1016 (Table 1).

For the sake of comparison, all primer/probe sets were assayed in the same conditions: real-time reactions were prepared in 25 µL volume using the TaqPath BactoPure Microbial Detection Master Mix (Thermo Fisher Scientific, https://www.thermofisher.com), 500 mmol of each primer, 250 nmol of each probe, and 5 µL of sample DNA. Amplification conditions included an initial activation at 95°C for 2 min, and 45 cycles of 10 s at 95°C and 30 s at 60°C. Each reaction was run in triplicate on a QuantStudio 12K Flex (Applied Biosystems-Thermo Fisher) (Appendix Table 1). The 3 primer/probe sets with the lowest quantification cycle (Cq) value were selected for further optimization: PCR identification nos. 1003 (F3L), 1004 (N3R), and 1005 (G2R).

Standardization of Monkeypox DNA provided by the European Virus Archive Global (EVAg)

To standardize material for the optimization and performance characterization of the selected real-time PCR, we quantified used Monkeypox virus (Slovenia ex Gran Canaria) DNA batch 06.06.2022 (European Virus Archive Global [EVAg]; https://www.european-virus-archive.com) the Monkeypox DNA (Monkeypox virus, 2022, Slovenia ex Gran Canaria, Ref. 005V-04714) by droplet digital PCR (ddPCR).

Tenfold dilutions of the DNA stock were prepared in molecular grade Tris-EDTA buffer (10 mM Tris, 1 mM EDTA) pH 8.0 (Sigma-Aldrich, https://www.sigmaaldrich.com). DNA

dilutions of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were tested in quadruplicate by ddPCR by using the QX200 system (Bio-Rad, https://www.bio-rad.com) and the ddPCR Supermix for probes kit without deoxyuridine triphosphate (dUTP) (BioRad). The reaction mixture included: 10 µL ddPCR supermix, primers 500 nmol, probes 250 nmol, and nuclease-free water to a final volume of 20 µL. Primers and probes were those described for PCR nos. 1003, 1004, and 1005. Droplets were generated as recommended by the manufacturer and amplification was performed on a 9600 GenAmp thermalcycler (Applied BioSystems) as follows: 95°C for 10 min, followed by 94°C for 30 s and 60°C for 60 s (40 cycles), and by a final stage at 98°C for 10 min. Results were acquired using the Bio-Rad QX200 Droplet Reader and QX Manager Standard Edition version 1.2 to provide absolute quantification of the target sequence (Appendix Table 2).

Because 2 copies of the G2R region are in MPXV genome due to its location at the ITR terminal regions, only the results of F3L and N3R were taken into account for the quantification of viral genome copies (copies/µL) of the Monkeypox Slovenia ex Gran Canaria, Ref-SKU: 005N-04716 provided by EVAg. Reference value was defined as the average of the values obtained for the 2 targets: 738,419 copies/µL = 7.4×10^5 copies/µL.

Optimization of Real-Time PCR Assays 1003 (F3L), 1004 (N3R), and 1005 (G2R) conditions

Testing of Different Real-Time Reagents

Based on previous experiences, the choice of real-time reagents may affect the efficiency of target virus detection in wastewater samples, due to different sensitivity of polymerases to factors as environmental inhibitors or supercoiling of target sequences. To select the most efficient reagents for Monkeypox DNA detection in wastewater, the standardized EVAg Monkeypox DNA was diluted in a 1:100 proportion in nucleic acids extracted from wastewater samples collected from urban WTPs in a period preceding the emergence of Monkeypox virus in Italy (November and December 2021).

Samples were then tested with the 3 PCR assays by using the following reagents: AgPath-ID One-Step RT-PCR Reagents (Applied Biosystems) for PCR 1003, TaqPath BactoPure Microbial Detection Master Mix (Thermo Fisher Scientific) for PCR 1004, and TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific) for PCR 1005.

All reactions were prepared in 25 µL volume using 500 mmol of each primer, 250 nmol of probe and 5 µL of sample DNA. AgPath-ID reactions included 1 µL/reaction of enzyme mix and 1.67 µL of detection enhancer. Reactions were run on a QuantStudio 12K Flex (Applied Biosystems).

The following amplification conditions were used to obtain Cq values (Appendix Table 3). For AgPath-ID One-Step RT-PCR Reagents, an initial reverse transcription inactivation step at 95°C for 5 min, followed by 45 cycles of 10 s at 95°C and 30 s at 60°C. For TaqPath BactoPure Microbial Detection Master Mix, an initial activation step at 95°C for 2 min, followed by 45 cycles of 10 s at 95°C and 30 s at 60°C. For TaqMan Fast Advanced Master Mix, an initial hold at 50°C for 2 min, followed by an activation step at 95°C for 20 s, and by 45 cycles of 10 s at 95°C and 30 s at 60°C. Because no significant difference was detected among the performance of the different reagents, we selected TaqPath BactoPure Microbial Detection Master Mix, because it required the least preparation time.

Primer/Probe Concentrations

Optimization of primer/probe concentrations was performed by testing the following concentrations in their different combinations, for each PCR assay (IDs 1003, 1004, and 1005): forward primer at 200 nmol, 500 nmol, 800 nmol; reverse primer at 200 nmol, 500 nmol, 800 nmol; and probe at 100 nmol, 250 nmol, 500 nmol. All reactions were prepared in 25 µL volume by using 5 µL of sample DNA (10^{-4} dilution of the standardized EVAg Monkeypox DNA) and the TaqPath BactoPure Microbial Detection Master Mix. Amplification conditions included an initial activation step at 95°C for 2 min, followed by 45 cycles of 10 s at 95°C and 30 s at 60°C. Reactions were run on a QuantStudio 12K Flex (Applied Biosystems), and graphically summarized results (Appendix Figure).

Based on Cq values, in all PCR assays, better amplifications were achieved at a probe concentration of 500 nmol and slightly better results were obtained with a primer concentration of 800 nmol. Although, in the primer, the differences with other concentrations were minimal (often >1 ΔCq). Therefore, concentrations of 500 nmol of probe and 800 nmol of primers were used for the analysis of the environmental samples.

Assessment of LOD_{50} for real-time PCR ID 1003 (F3L), 1004 (N3R) and 1005 (G2R)

LOD_{50} on Pure Target (Monkeypox DNA Diluted in TE buffer)

To assess the sensitivity of the real-time PCR assays used in the study, the LOD_{50} of each reaction was calculated according to Wilrich and Wilrich (https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html). We prepared 2-fold dilutions of the standardized EVAg Monkeypox DNA in molecular grade TE buffer pH 8.0 starting from the 10^{-5} dilution (7.4 copies/µL). Each dilution was tested in 8 replicates with PCR 1003 (F3L), PCR 1004 (N3R), and PCR 1005 (G2R) by using the optimized reaction conditions (Appendix Table 4).

LOD_{50} on Target in Wastewater (Monkeypox DNA Diluted in Nucleic Acid Extracted from Wastewater Samples)

To assess the sensitivity of the real-time PCR assays in the condition of use (i.e., detecting monkeypox virus in wastewater samples), the LOD_{50} of each reaction was also calculated, using the same approach described above, by testing the target monkeypox DNA diluted in nucleic acid extracted from wastewater samples, which include potential inhibitors of the polymerization reaction.

We prepared 2-fold dilutions of the standardized EVAg Monkeypox DNA in molecular grade TE buffer pH 8.0 starting from the 10^{-4} dilution (74 copies/µL). Each dilution was then used to spike 1:10 proportion nucleic acid extracted from wastewater samples collected from urban wastewater treatment plants during November–December 2021, prior to emergence of monkeypox virus in Italy. Spiked samples were tested in 8 replicates with PCR 1003 (F3L), PCR 1004 (N3R), and PCR 1005 (G2R), using the optimized reaction conditions (Appendix Table 5).

Appendix Table 1. Primers used to detect monkeypox virus DNA in airport wastewater, Rome, Italy*

PCR ID	Target	Primer name	Primer ID	Average Cq	SD
1002	G2R	MPVX G F	2368	23.28†	0.55
		MPVX G R	2369		
		MPVX G P	2370		
1005	G2R	MPVX G F mod	2377	21.93	0.25
		MPVX G R mod	2378		
		MPVX G P	2370		
1003	F3L	F3L-F290	2371	22.95	0.31
		F3L-R396	2372		
		F3Lp333S-MGB	2373		
1008	F3L	F3L-F290	2371	23.56	0.25
		F3L-R396 mod	2384		
		F3Lp333S-MGB	2373		
1004	N3R	N3R-F319	2374	21.76	0.16
		N3R-R457	2375		
		N3Rp352S-MGB	2376		
1016	N3R	N3R-F319	2374	26.69†	0.43
		N3R-R457	2375		
		N3Rp352S-MGB mod	2381		

*Cq, quantification cycle; ID, identification; F, forward; mod, modified; MPXV, monkeypox virus; P, probe; R, reverse.
†Duplicate reaction due to run setting failure.

Appendix Table 2. Control DNA used in 3 PCR assays to detect monkeypox virus DNA in airport wastewater, Rome, Italy*

Dilution	PCR 1005, G2R	PCR 1003, F3L	PCR 1004, N3R
10^{-2}	NA†	7,665.25	6,973.25
10^{-3}	1,171.5	751.75	739.25
10^{-4}	120	76.5	78.75
10^{-5}	16	7.25	6.75
Weighted average	13,238.33	7,520.69	7,247.69
Stock DNA, copies/µL	1.3×10^{6}	7.5×10^{5}	7.2×10^{5}

*Controls expressed as copies/µL. NA, not achieved.
†Suboptimal separation of positive and negative events

Appendix Table 3. Reagents used in 3 PCR assays to detect monkeypox virus DNA in airport wastewater, Rome, Italy*

Reagent	PCR 1005, G2R	PCR 1003, F3L	PCR 1004, N3R
AgPath-ID One-Step RT-PCR	21.71	23.07	21.60
TaqPath BactoPure Microbial Detection Master Mix†	21.90	23.18	21.91
TaqMan Fast Advanced Master Mix	22.20	22.60	21.78

*Reagents available from Thermo Fisher Scientific, https://www.thermofisher.com.

Appendix Table 4. Target concentrations and limits of detection used to detect monkeypox virus DNA in airport wastewater, Rome, Italy*

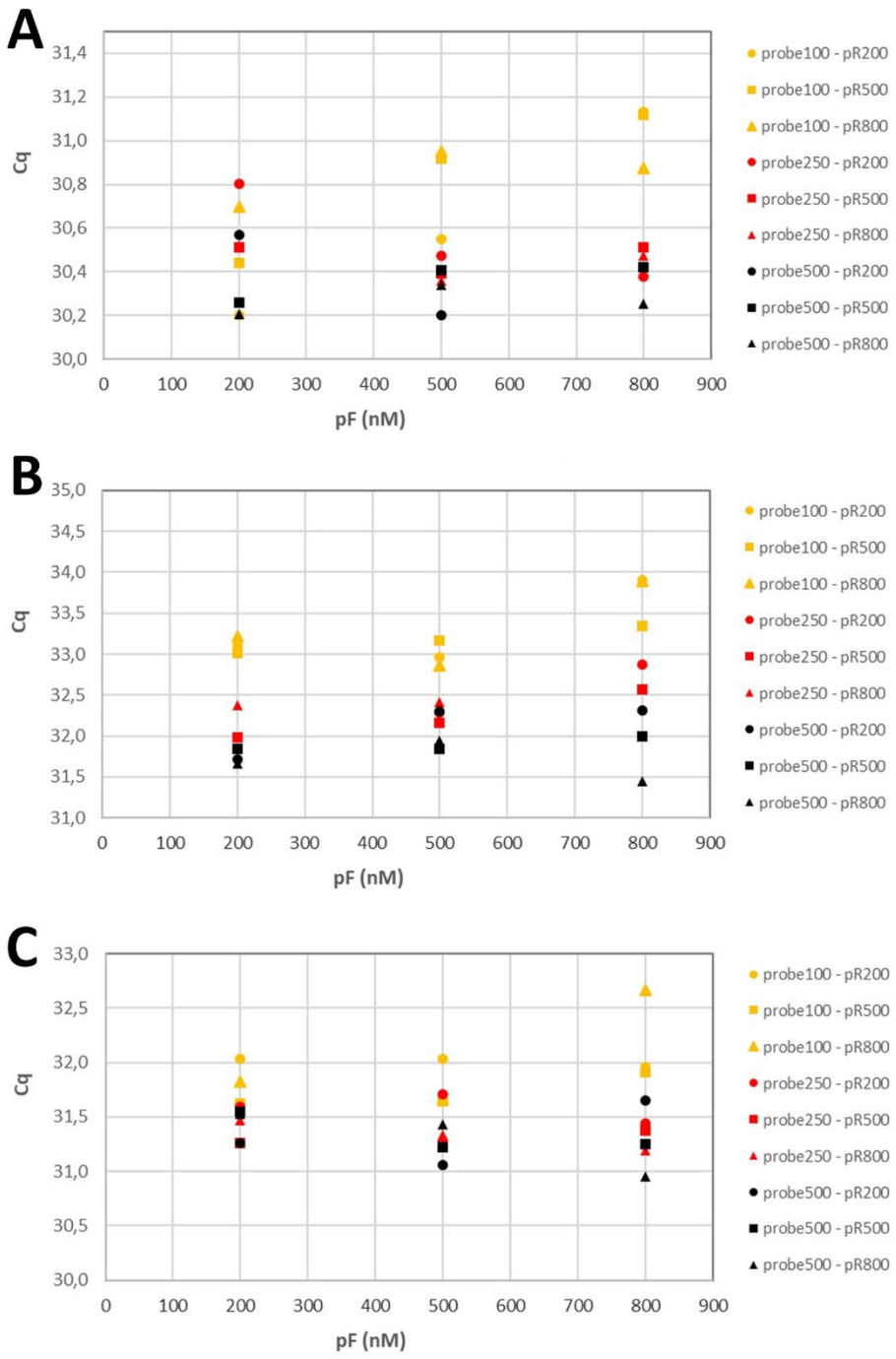
Concentration and limit of detection	PCR 1005, G2R	PCR 1003, F3L	PCR 1004, N3R
Expected target concentration, copies/µL			
3.7	8/8	8/8	8/8
1.85	8/8	8/8	8/8
0.925	7/8	8/8	8/8
0.4625	7/8	5/8	7/8
0.23125	4/8	2/8	3/8
0.115625	3/8	1/8	2/8
Limit of detection			
LOD_{50} copies/µL	0.213	0.306	0.206
LOD_{95} copies/µL	0.923	1.323	0.890
LOD_{50} copies/reaction	1.065	1.530	1.030
LOD_{95} copies/reaction	4.615	6.615	4.450

*Results are expressed as no. positive/no. analytic replicates. LOD_{50}, limit of detection for which the probability of detection is 50%; LOD_{95}, limit of detection for which the probability of detection is 95%.

Appendix Table 5. Target concentrations and limits of detection used to detect monkeypox virus DNA in airport wastewater, Rome, Italy*

Concentration and limit of detection	PCR 1005, G2R	PCR 1003, F3L	PCR 1004, N3R
Expected target concentration, copies/µL			
7.4	8/8	8/8	8/8
3.7	8/8	8/8	8/8
1.85	8/8	8/8	8/8
0.925	6/8	7/8	7/8
0.4625	3/8	6/8	5/8
0.23125	3/8	1/8	3/8
Limit of detection			
LOD_{50}, copies/µL	0.432	0.330	0.309
LOD_{95}, copies/µL	1.868	1.427	1.336
LOD_{50}, copies/reaction	2.160	1.650	1.545
LOD_{95}, copies/reaction	9.340	7.135	6.680

*Results are expressed as no. positive/no. analytic replicates. LOD_{50}, limit of detection for which the probability of detection is 50%; LOD_{95}, limit of detection for which the probability of detection is 95%.



Appendix Figure. Optimization of primer/probe concentrations used to detect monkeypox virus DNA in airport wastewater, Rome, Italy. Optimization was performed by testing concentrations in their different combinations for each of 3 PCR assays. A) PCR 1003 for F3L; B) PCR 1004 for N3R; and C) PCR 1005 for G2R.